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REVIEW ARTICLE

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The equine mononuclear phagocyte system: The relevance of the horse as a model for understanding human innate immunity

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Abstract

The mononuclear phagocyte system (MPS) is a family of cells of related function that includes bone marrow progenitors, blood monocytes and resident tissue macrophages. Macrophages are effector cells in both innate and acquired immunity. They are a major resident cell population in every organ and their numbers increase in response to proinflammatory stimuli. Their function is highly regulated by a wide range of agonists, including lymphokines, cytokines and products of microorganisms. Macrophage biology has been studied most extensively in mice, yet direct comparisons of rodent and human macrophages have revealed many functional differences. In this review, we provide an overview of the equine MPS, describing the variation in the function and phenotype of macrophages depending on their location and the similarities and differences between the rodent, human and equine immune response. We discuss the use of the horse as a large animal model in which to study macrophage biology and pathological processes shared with humans. Finally, following the recent update to the horse genome, facilitating further comparative analysis of regulated gene expression between the species, we highlight the importance of future transcriptomic macrophage studies in the horse, the findings of which may also be applicable to human as well as veterinary research.

KEYWORDS
horse, macrophage, monocyte, immunity, animal model

1 | INTRODUCTION

Horses are already recognised as models for several human diseases, including metabolic syndrome, asthma, musculoskeletal diseases, melanoma and autoimmune uveitis.[1-6] More than 100 equine hereditable conditions may serve as models for human disorders, including inflammation, muscular or fertility disorders, osteoarthritis and even depression.[7-10] The horse has the potential to represent an appropriate

Abbreviations: AM, alveolar macrophage; BAL, bronchoalveolar lavage; BMDMs, bone marrow-derived macrophages; CSF1, colony-stimulating factor 1; CSF1R, colony-stimulating factor 1 receptor; CSF2, colony-stimulating factor 2; CXCL10, C-X-C motif chemokine ligand 10; FLT3L, FMS-like tyrosine kinase 3 ligand; GIT, gastrointestinal tract; ICC, interstitial cells of Cajal; IFN, interferon; IL-10, interleukin-10; IMs, interstitial macrophages; LpM, lamina propria macrophages; MIP-2, macrophage inflammatory protein 2; MM, muscularis macrophages; MMEA, mild to moderate equine asthma; MP, myenteric plexus; MPS, mononuclear phagocyte system; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PIMs, pulmonary intravascular macrophages; PM, peritoneal macrophage; POI, post-operative ileus; Poly IC, polyinosinic polycytidylic acid; PRR, pattern recognition receptor; SEA, severe equine asthma; TRAF1, TNF receptor-associated factor 1; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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large animal model in which to study basic macrophage biology and pathological processes shared with humans, with the advantage that insights can be applied in both veterinary and human medicine.

Monocytes and macrophages provide the first line of defence against pathogens and play a crucial role in both health and disease.[11] Macrophage biology has been studied most extensively in mice, with the data derived from such studies having undoubtedly helped to unravel basic physiological mechanisms in health and disease processes. However, direct comparison of mouse and human macrophages has revealed many functional differences.[12,13] In contrast, important similarities have already been described between horse and human macrophages.[14-22] In this review, we provide an overview of equine macrophage biology and highlight the importance of future macrophage studies in the horse, the findings of which may also be applicable to man.

2 | AN OVERVIEW OF MACROPHAGE BIOLOGY AND THE MONONUCLEAR PHAGOCYTE SYSTEM IN HUMANS AND RODENTS

Macrophages (from Greek 'μακρόν' meaning big and 'φαγείν' meaning to eat) are large leucocytes that comprise 10%-15% of all cells in most organs of all animal species.[23-25] They were originally identified in the 19th century by Metchnikoff, who distinguished them from related microphages, now known more commonly as granulocytes or polymorphonuclear leucocytes. Subsequently, van Furth and Cohen and others recognised the functional relationship between bone marrow progenitors, circulating blood monocytes and resident tissue macrophages and proposed the concept of a mononuclear phagocyte system (MPS).[26,27] The shared functions of cells of the MPS include antigen presentation, phagocytosis, cytokine production, microbicidal activity, tissue repair and the general regulation of tissue homeostasis (Figure 1).

Over the past 5 years there have been numerous studies on the development of the MPS in the mouse. Macrophage-like cells first

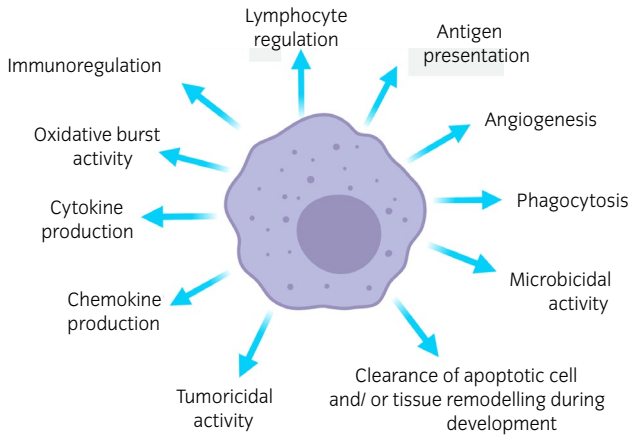

FIGURE 1 Summary of macrophage functions

appear in the yolk sac without an evident monocyte-like intermediate, and thence are produced in the fetal liver (reviewed in 28,29). Furthermore, lineage-trace studies suggest that many tissue macrophage populations (the notable exception being the large macrophage population of the intestinal mucosa) are derived from fetal progenitors and maintained in the steady state without substantial input from blood monocytes.[30-35] The tissue itself plays an important role in controlling the balance between the persistence of the resident macrophage population and the recruitment of circulating monocytes at various stages of its maturation.[31] Humans with mutations in the IRF8 transcription factor lack blood monocytes, yet have tissue macrophages.[36] Although these results are often portrayed as a contradiction to the MPS concept, the original definition of the MPS clearly did recognise the ability of resident macrophages to self-replicate.[29] Many studies in multiple organ systems have shown that blood monocytes can, and do, occupy the vacant territories of resident macrophages when these cells are depleted (reviewed in 28,29,31,37). It is important to recognise that the lineage-trace models that favour the persistence of fetal-derived macrophages are open to interpretation.[29,37] Furthermore, the recent concepts of MPS ontogeny and homeostasis are derived mainly from a single inbred mouse strain (C57Bl6) under specified pathogen-free laboratory conditions. It is not yet clear that the conclusions derived from these studies can be applied even to other mouse strains let alone other species, including the horse.[29,37,38]

Although all tissue macrophages share many surface and intracellular markers and a characteristic ramified or stellate morphology, they also adapt their function and gene expression to perform specific functions. For example, hepatic and splenic macrophages, which have direct contact with the blood, are adapted for erythrophagocytosis and recycling of iron, whereas a population of bone marrow macrophages is adapted to support erythropoiesis in erythroblastic islands.[39] Embryonic macrophages have a common gene expression profile, with tissue-specific macrophage adaptation arising mainly in the early postnatal period, in parallel with cessation of the major proliferative phase and appearance of organ-specific functions.[40,41] Adaptation is driven in part by expression of specific transcriptional regulators.[40] For example, two recent studies described in detail the adoption of liver macrophage (Kupffer cell)-specific gene expression profiles by monocytes recruited following Kupffer cell depletion, thus emphasising the roles of transcription factors *Id3* and *Nr1h3*.[42,43] Macrophages may even adapt to multiple specific niches within tissues. For example, Chakarov *et al*. noted that subpopulations of macrophages expressing the surface marker *Lyve1* are associated specifically with capillaries in the lung and several other tissues.[44] Figure 2 summarises some of the specific subpopulations that have been described in mouse tissues. Recent studies of tissue-specific macrophage populations are reviewed in Hume et al.[29]

Whether or not these resident macrophage populations are replenished by monocytes in the steady state, monocytes are recruited to tissues in response to inflammatory stimuli. The gene

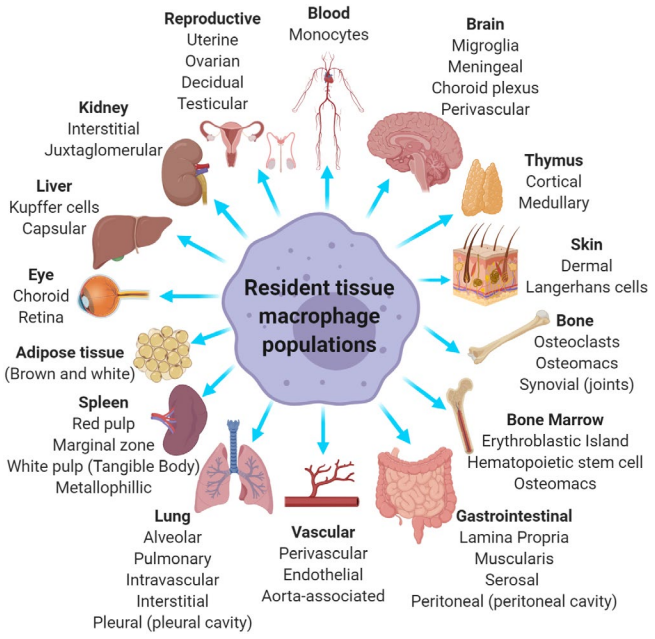

FIGURE 2 Main types of cells comprising the mononuclear phagocyte system in adult mammals

expression profiles of inflammatory monocytes/macrophages differ depending upon the nature of the inflammatory stimulus and change with time as the stimulus is removed and normal homeostasis is restored. Based upon a parallel with the Th1/Th2 dichotomy in T cells, there is a prevalent view that macrophage 'activation' can be divided into M1 or classical (mediated by the Th1 lymphokine, INFG) or M2 or alternative (mediated by the Th2 lymphokines, IL-4/IL-13). This dichotomy is not supported by transcriptomic analysis, which also reveals that few markers of M1/M2 divergence are conserved from mouse to human.[11,45,46] Recent reviews support a much wider spectrum of activation states and also the concept of innate immune memory, wherein macrophages retain an epigenetic imprint of previous exposure to a stimulus such as microorganisms.[47]

The development and maintenance of the MPS depends upon macrophage colony-stimulating factor 1 receptor (CSF1R) and its two ligands, macrophage colony-stimulating factor 1 (CSF1, also known as M-CSF) and interleukin 34 (IL-34). Mutations of the *Csf1r* in mice, rats and humans are associated with global deficiencies of tissue macrophage populations and multi-system developmental abnormalities, including severe postnatal growth retardation (reviewed in 48). Many of these deficiencies manifest in mice and rats with *Csf1* mutations, whereas IL-34 appears to be involved specifically in the development of macrophages of the brain (microglia) and skin (Langerhans cells).[49] The requirement for *Csf1r* for survival of resident macrophages is retained in adults and blocking CSF1R leads to progressive depletion of most macrophage populations.[50,51] Conversely, treatment of mice, rats or pigs with recombinant CSF1 leads to a monocytosis as well as proliferative expansion of resident macrophage populations.[52-55] These findings, combined with the fact that macrophages of the liver and spleen are the main site of clearance of CSF1, led to the concept of homeostatic control of the entire MPS via the availability of CSF1.[37] The exception to this concept is the lung, where another growth factor, granulocyte-macrophage colony-stimulating factor 2 (CSF2, also known as GM-CSF), is uniquely required for both development and resident macrophage homeostasis.[56]

In this review, we will describe the most studied components of the MPS of the horse (Figure 3) and highlight the effect of the microenvironment on macrophage function and phenotype. Finally, we will discuss the potential value of the horse in providing a greater understanding of macrophage biology in humans.

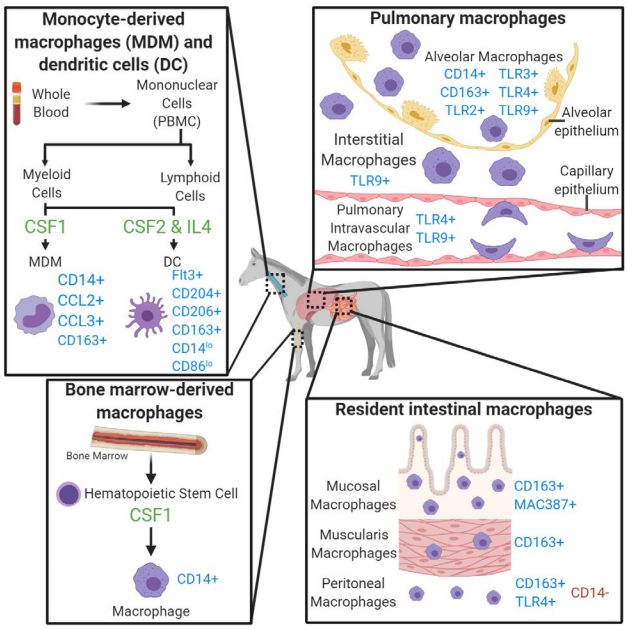

FIGURE 3 Monocyte and macrophage populations in the horse. This figure illustrates key monocyte and macrophage populations based on studies in the horse. Macrophages in horses have been identified by a combination of cell surface markers, including CD14 and CD163, although studies and reagents are limited in number. Macrophages in different tissues express different levels of cell surface proteins highlighting the diversity in resident macrophage populations. Bone marrow-derived macrophages, which are cultured from haematopoietic progenitor cells collected from bone marrow, express CD14. Monocytes isolated from peripheral blood mononuclear cells (PBMC) using a density gradient express CD14, CCL2 and CCL3, whereas monocyte-derived dendritic cells cultured in CSF2 and IL-4 express only low levels of CD14 and CD86 compared to monocytes, but express the dendritic cell markers FLT3 and CD206. Other markers used to characterise equine dendritic cells include CD172a MHC class II, CD44, CD163, CD204 and Bla36 (*not shown in figure*). Intestinal and peritoneal macrophages all express CD163. It is not known if intestinal macrophages express CD14 in the horse, although peritoneal macrophages do not. Alveolar macrophages express CD14, CD163, TLR2, TLR3, TLR4 and TLR9, while pulmonary intravascular macrophages have high expression of TLR4 and TLR9. Equine pulmonary interstitial macrophages have been shown to express TLR9 by immuno-electron microscopy

3 | THE MONONUCLEAR PHAGOCYTE SYSTEM OF THE HORSE

3.1 | Bone marrow-derived macrophage differentiation and activation in the horse

Bone marrow-derived macrophages (BMDMs) are primary macrophage cells generated in cell culture. They are derived from progenitor cells and in the presence of the macrophage lineage-specific growth factor CSF1 they differentiate and proliferate into macrophages.[57] In mice, macrophages are routinely generated in vitro from bone marrow progenitors for subsequent functional studies.[58,59] This approach has recently been extended to large animal species expedited by the capacity to freeze cells for future culture following recovery.[21,60-62] We have recently published a comparative analysis of RNA-seq data of horse BMDM and their response to lipopolysaccharide (LPS) with similar data for ruminants (sheep, goat, cow and water buffalo), pig, mouse and rat (in press). The analysis reveals conservation of the underlying transcription factor repertoire, basal macrophage-specific gene expression and LPS-inducible cytokines. However, all of the large animals are clearly distinguished from rodents, and horse macrophages have a small set of species-specific macrophage-expressed transcripts that will be the subject of future studies.

Similar to other groups,[63-65] we have also differentiated equine monocytes to macrophages in the presence of horse serum which, among other stimuli, contains CSF1 (A.E. Karagianni, 2014, unpublished data). Bone marrow colony assays have been described using horse marrow and used to study the transforming actions of equine infectious anaemia virus.[66] Furthermore, a macrophage cell line (e-CAS cells) has been derived from equine bone marrow cells[67] and subsequently shown to have phagocytic capabilities and responsiveness to CSF2 and LPS. However, potential problems associated with cell lines include contamination by other cells or microorganisms,[68] which can result in molecular and cellular changes in the cell line.[69] These problems have very recently been highlighted by Evans et al,[70] who sequenced the e-CAS cell line and found the cells to likely be of mouse rather than horse origin. Finally, equine bone marrow-derived mononuclear cells have also been used in cell therapy methodologies in horses affected with equine asthma, whereby intratracheal administration ameliorates the inflammatory response in the lungs, resulting in beneficial effects on clinical signs.[71] Similar observations have already been reported in mice.[72]

3.2 | Monocyte differentiation and activation in the horse

Based mainly on murine literature, during postnatal life, monocytes can replace resident macrophages in all major organs and acquire their gene expression profile.[29] They share the expression of several surface markers with macrophages and together with dendritic cells (DCs), act as antigen-presenting cells and play a vital role during infection.[29] Several studies in multiple organs have found that blood monocytes can occupy the vacant territories of tissue macrophages when these populations are depleted (reviewed in 28,29,31,37). In the horse, similar to other well-studied species, blood is more easily sampled than bone marrow. Consequently, several studies have been conducted on equine monocytes isolated from peripheral blood mononuclear cells (PBMC), focusing on their ability to produce proinflammatory cytokines, such as TNF and IL-1, under various conditions, including LPS stimulation.[73-79] Raabe et al[64] also demonstrated the transforming actions of equine infectious anaemia virus on equine blood monocytes. Others have shown that equine peripheral blood monocytes and monocyte-derived macrophages express CXCL10, a Th1 marker, in response to IFN-γ stimulation.[80,81] The induction of equine CXCL10 by IFN-γ is also in agreement with human and murine studies.[82,83]

Compared to alveolar macrophages (AMs), equine PBMC appear more sensitive to low concentrations of LPS, a finding which may reflect desensitisation of AMs by chronic low-level stimulation with inhaled proinflammatory agents.[84] In humans and mice, the macrophage response to LPS can be mediated via two different pathways; a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway, which induces an inflammatory response, and/or a MyD88-independent pathway (also known as the TIR-domain-containing adapter-inducing interferon-β [TRIF] pathway), which stimulates the induction of type I interferon (*IFN*) and *IFN*-inducible genes.[85,86] These pathways appear to be conserved in horses (Figure 4). In equine macrophages/monocytes, the induction of genes encoding *TNF*, *IL1B* and *IL6* was linked to activation of the MyD88 pathway, whereas *IFNA*, C-X-C motif chemokine ligand 10 (*CXCL10*) and *RANTES* (also known as *CCL5*) were related to the TRIF-dependent signalling pathway.[87] Even high concentrations of LPS failed to significantly activate TRIF-dependent gene expression of *IFN*, *CXCL10*, *RANTES* or *TNF* receptor-associated factor 1 (*TRAF1*) in equine monocytes, suggesting that in contrast to other studied mammalian-derived cells, the response of equine monocytes to LPS mainly occurs via the MyD88 pathway.[87] Furthermore, Ahn et al[88] studied the response of equine PBMC to well-known inflammasome activators in other species (including human and mouse), whereby equine PBMC were shown to secrete IL-1B, a well-known indication of inflammasome activation. Based on comparative studies, the authors indicated that equine inflammasome activation is similar to that in humans, mice and pigs.

As demonstrated in other mammalian-derived cells, TLR2 ligands induce a mild inflammatory response in equine PBMC; this is in comparison to the much greater response to TLR4 agonists.[9,89] Recent transcriptomic studies have reported that LPS induces differential microRNA expression in equine PBMC in a manner comparable to humans, thus facilitating interspecies comparative study of the role of microRNAs in the inflammatory cascade during endotoxaemia and sepsis.[90] Equine monocytes are also responsive to TLR3 stimulation with double-stranded RNA (polyinosinic polycytidylic acid [Poly IC]),[91] which is dependent upon the TRIF adaptor. In contrast, horse monocytes do not express TLR5, thus explaining their lack of response to flagellin.[92] However, they do express the scavenger

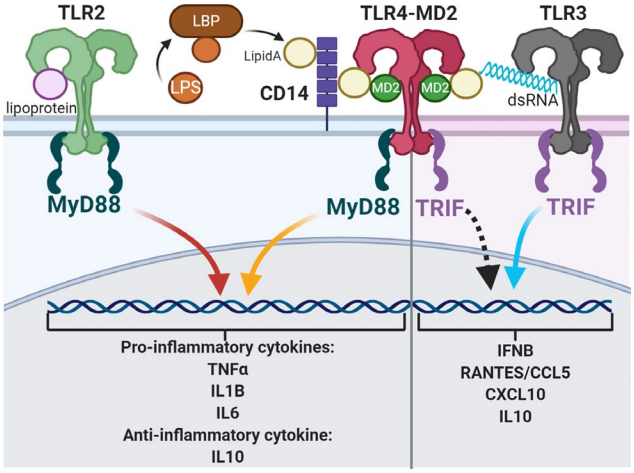

FIGURE 4 TLR 2, 3 and 4 signal transduction pathways of the horse. Recognition of lipoprotein ligand by TLR2 triggers the MyD88-dependant signalling pathway (red arrow). Following stimulation with LPS, lipopolysaccharide binding protein (LBP) is recruited to transfer LPS via the Lipid A component to CD14 on the surface of macrophages/monocytes. This process is followed by the formation of a complex with TLR4 and MD2, activation of NFKB signalling pathway and cytokine production. In humans and mice, the recognition of the LPS ligand results in a conformational change in the TLR4 receptor triggering a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and/or a MyD88-independent pathway (also known as the TIR-domain-containing adapter-inducing interferonβ (TRIF) pathway). Equine monocytes fail to strongly activate the TRIF pathway in response to LPS (black dashed arrow); instead, they respond mainly via the MyD88-dependent pathway (yellow arrow), resulting in the induction of the proinflammatory cytokines *TNF*, *IL1B*, *IL6* and the anti-inflammatory cytokine *IL10*. In contrast, TLR3 signalling occurs via the TRIF pathway (blue arrow), inducing high expression of IFNB, CXCL10 and RANTES

receptor CD163,[93] as do equine alveolar and peritoneal macrophages (PMs).[20] Moreover, equine monocytes isolated from PBMC using a density gradient express CD14, CD16, CCL2 and CCL3.[16,94] Unlike humans and horses, mice do not have duplicated CD16 gene, while $CD14^{+}CD16^{+}$ have also been detected in humans and horses but not in mice.[16]

The MPS includes antigen-presenting DCs. The term DC is loosely applied to any cell that can present antigen to naïve T cells, including bone marrow or monocyte-derived cells cultivated in CSF2. However, there has been increasing recognition that these 'DCs' are distinct from those generated in response to FMS-like tyrosine kinase 3 ligand (FLT3L), a cytokine essential for their development; are derived from a committed DC progenitor[95] and may be better considered as an alternative state of macrophage differentiation.[37]

Monocyte-derived equine DCs[96] have also been generated in various studies by cultivation in CSF2 and found to express different cell markers including FLT3, CD86, CD204, CD206, CD163 and low levels of CD14.[97-100] Moreover, horse microarrays were used to investigate the gene profile of equine immature and mature DC subsets, confirming the existence of two clearly distinct populations.[97] In contrast, comparisons between lung and blood-derived DC of the horse showed no morphological differences.[101] However, blood-derived DCs expressed higher levels of CD86 and CD172α than those in the lung, while both expressed MHC class II and CD44 and, to a lower level, the scavenger receptors CD163, CD204 and the B lymphocyte antigen Bla36.[101] Furthermore, lung DC demonstrated higher phagocytic activity compared to their blood-derived counterparts.[101] Interestingly, others have also reported remarkable age-dependent phenotypic and functional differences between blood monocyte-derived DCs from foals and adult horses.[102] Following bacterial exposure, DCs generated from foals exhibited lower antigen presentation capabilities and produced lower quantities of proinflammatory cytokines. In summary, these results highlight the influence of both cell origin and ageing on both phenotypic and functional characteristics.

3.3 | The role of the equine resident macrophages in intestinal disease

3.3.1 | Resident intestinal macrophages

The gastrointestinal tract (GIT), although internal, has the largest surface area in contact with the external environment of all the body organs and thus receives a vast and constant antigenic challenge. It is therefore not surprising, based on data derived from humans and rodents, that the GIT represents the largest reservoir of mononuclear phagocytes in the body.[103] They are located throughout all the tissue layers of the intestine and are broadly categorised into two phenotypically distinct groups: mucosal or lamina propria macrophages (LpM) and *muscularis* macrophages (MM).

Lamina propria macrophages are found in the mucosa of the GIT, predominantly in the lamina propria,[104] and in both mice and humans express CD64, MHC class II, CD206 and CD163. In contrast to human LpM, mouse LpM also express CD11b, CD11c and CX3CR1.[105] Given their close proximity to the intestinal lumen and the potential for constant antigenic challenge, it is not surprising that, unlike other tissue macrophages, LpM appear to be in a state of 'hyporesponsiveness'.[106] In humans, this has been attributed to the absence of expression of the LPS receptors CD14[107] and TLR4,[108] the downregulation of TLR2,[108] their inability to produce proinflammatory cytokines, such as TNFα, and the production of the anti-inflammatory cytokine, interleukin-10 (IL-10).[109,110] In contrast, MM, which are located in the serosa, myenteric plexus and muscle layers of the mouse and human GIT,[111,112] do express CD14[113] and TLR4[114] and may participate in endotoxin-mediated responses within the muscularis. MM in mice and humans also express CD163, MHC class II, CD206 and CD64 with two different subtypes proposed, depending on the state of activation.[105,115]

Both LpM and MM survival is dependent upon signalling from the CSF1R.[51,116] Depletion of LpM using an anti-CSF1R antibody resulted in a disruption to the differentiation and proliferation of intestinal epithelial cells,[117] while depletion of MM resulted in disruption

of peristalsis in mice, thus demonstrating a role of MM in intestinal motility.[116] Studies in mice have shown that MM have a slower turnover rate than LpM;[112] although both are derived from both yolk sac and monocytes,[35] LpM in both mice and humans are continuously replaced by blood monocytes.[118,119] This monocyte infiltrate is also important for tissue repair following inflammation, as demonstrated in a mouse model of post-operative ileus (POI).[120] CD163 has been used to identify mucosal and MM in the horse.[121-123] MAC387 has also been used to identify mucosal macrophages in horses, although this nonspecific macrophage marker is also expressed by granulocytes.[124,125] $CD163^+$ macrophages are present in all tissue layers of the equine small and large intestine, although the distribution of cells is not uniform.[123] Different cell densities were observed between the different tissue layers (lamina propria, submucosa and muscularis externa). Within the submucosa, there was also a higher density of $CD163^+$ cells in the large intestine compared to the small intestine. This increase in density of $CD163^+$ cells could be a consequence of the higher increase of bacterial content in the intestinal lumen of the large intestine compared to the small intestine.

The role of macrophages in humans and rodents has been clearly demonstrated in various intestinal pathologies of the GIT, particularly in POI and inflammatory bowel disease (IBD).[126-129] Despite both these conditions occurring in horses (reviewed in 130 and 131), the study of macrophage responses in equine intestinal disease is very limited. The identification of macrophages in the cellular infiltrate of equine IBD cases is a criteria considered in the sub-categorisation of disease; macrophage infiltrate is associated with granulomatous enteritis and multisystemic eosinophilic epitheliotropic disease but not with lymphocytic-plasmacytic enterocolitis or idiopathic eosinophilic enterocolitis.[130] However, these cellular-based disease sub-categories are generally based on haematoxylin and eosin staining; to date, no studies have used specific markers to study different macrophage subpopulations in horses, as has been done in mice[129] and humans.[128] Although Little et al[132] used calprotectin as a macrophage marker in an equine model of intestinal manipulation; calprotectin is also expressed in neutrophils and monocytes. This study did demonstrate an intestinal manipulation-induced local inflammatory response in horses, similar to that of rodents and humans which ultimately leads to smooth muscle dysfunction and POI.[126,127] Intestinal ischaemia and reperfusion in horses results in an increased phagocytic activity of $CD163^+$ macrophages without a change in numbers, suggesting their potential role in the resolution of inflammation[121] and supporting CD163 as a macrophage marker for pro-repair (or M2) macrophages.[133] Thus, CD163 expression in resident equine intestinal macrophages[123] suggests that the normal equine GIT is populated with intestinal macrophages associated with an anti-inflammatory phenotype. By contrast, human studies have demonstrated a contribution of $CD163^+$ cells to the amplification of inflammation in the mucosa in IBD patients.[134] Furthermore, the proinflammatory mediators, IFN-γ, LPS and TNF, are found to suppress the expression of CD163 in human monocytes,[135] suggesting that CD163 may be regulated by both pro- and anti-inflammatory mediators. Given the relative sparsity of studies of macrophage populations of the intestine of the horse, it is difficult to draw conclusions. There remains a need for further study of immune cell populations in the equine GIT and development of additional markers.

3.3.2 | Equine peritoneal macrophages

The peritoneal cavity is another major source of macrophages for functional studies in rodents, in many cases following injection of an irritant or infectious agent.[136] This anatomical compartment has also been a commonly used source of macrophages in mice. In this species, resident PMs have a unique expression profile dependent upon the transcription factor, *Gata6*.[137,138] PMs have also been isolated and studied in humans[139] and pigs.[140] The function of peritoneal-derived macrophages is likely to be influenced by their close proximity to the GIT, potentially acting as a defence against any breach of the integrity of the intestinal wall which may result in exposure to intestinal luminal-derived microflora and various bacterial-derived products.[141] One of the genes that is highly expressed in resident macrophages in mice and humans is *Vsig*, encoding the surface receptor CrIG. In humans, CrIG-positive macrophages in the peritoneum are associated with disease severity and susceptibility to infection in patients with liver disease and ascites.[142,143]

Peritonitis is a significant clinical issue in horses, most commonly associated with surgery for indications such as colic.[144] LPS stimulation of equine PMs induced the significant production of prostanoids and proinflammatory cytokines,[145-147] but did not produce nitric oxide (NO).[17] This LPS-induced response could be suppressed by high concentrations of dexamethasone, as well as by IL-10.[17,148] Interestingly, LPS-induced TNF production from PMs harvested from healthy horses was significantly increased compared to PMs derived from horses with acute gastrointestinal disease, leading to the suggestion that PMs harvested from the latter group may have developed early endotoxin tolerance in response to disease-associated endotoxaemia.[149] However, in this study there was marked horse-to-horse variation, and the authors relied on measuring TNF bioactivity rather than protein expression.

We have also reported on the failure of equine PMs, immediately isolated from euthanised horses, to produce TNF in response to LPS, Poly IC and heat-killed *S. typhimurium* stimulation. This cell population expressed both CD163 and TLR4, but not CD14.[20] Other studies also reported that the LPS responsiveness of equine PMs was neither dose- nor time-dependent, as assessed by a TNF bioassay.[17,146,148] Previous studies on the LPS responsiveness of equine PMs used cells harvested from live horses, whereby peritoneal fluid is collected from the most dependent abdominal site of standing subjects, likely containing a PM population which has gravitated at this location.[17,146,148] In contrast, the collection procedure we adopted to harvest PMs from euthanased horses involved high volume lavage of the entire abdominal cavity, potentially resulting in the isolation of a PM population more representative of the entire abdominal cavity including those loosely adherent to the peritoneum. Finally, site-dependent differences in the differential gene expression of

AMs and PMs derived from euthanased horses also supported the influence of the tissue microenvironment on macrophage function and phenotype.[22]

3.4 | Pulmonary macrophages: key players in the immune defence of the equine lung

At least three types of macrophages have been identified so far in the lungs: the AMs, the interstitial macrophages (IMs) and the pulmonary intravascular macrophages (PIMs). PIMs are absent in healthy humans and mice, but are present in horse, sheep and other species.[150,151] Published studies, mainly in mice, have differed in their conclusions with respect to the existence of significant functional differences between these populations of lung macrophages.[152-156] As bronchoalveolar lavage (BAL) is a practical and commonly used method of obtaining resident airway cells, there are several published transcriptome analyses of BAL-derived AMs in different species, including large animals such as pigs,[157] sheep[61] and horses.[22] They are clearly distinct from other tissue macrophages, expressing many lectin-like receptors apparently adapted to recognise, internalise and eliminate inhaled microorganisms. The resident AMs arise during embryogenesis and self-renew throughout life without major replacement from circulating monocytes.[32] However, yolk sac-derived monocytes/macrophages, blood monocytes or bone marrow transplanted into *Csf2r*$^{-/-}$ mice can re-establish the missing population and successfully differentiate to AMs.[158]

In comparison, IMs are located in the bronchial and not the alveolar interstitium and more closely resemble blood monocytes and are therefore clearly distinct from AMs.[151,156] Moreover, recent transcriptomic studies also described three newly identified IM subsets in the murine lung at steady state.[156] Despite the functional, morphological and transcriptional differences between these two cell types, both are essential for lung homeostasis and immune responses.[151,154]

Although most equine-based studies have focused on the function of AMs and their response to various stimuli, more recently, Lee et al[101] studied the different subpopulations of myeloid cells in equine lung tissue. This latter study highlighted the differences and similarities in the function and phenotype of lung and blood myeloid cells. Both interstitial and AMs are thought to contribute to airway inflammation. For example, in severe equine asthma (SEA), a common environmental respiratory disease in adult horses, certain hay dust-derived components, including endotoxin and fungal spores, activate macrophages, resulting in the induction of chemokines, such as IL-8 and macrophage inflammatory protein 2 (MIP-2) or CXCL2[159,160] and the subsequent recruitment of neutrophils to the airway.[161-167] This neutrophil accumulation in turn leads to free radical and protease-mediated tissue damage, a process also seen in human studies.[168-170]

Respiratory infections or airway inflammation are very common in horses; even subtle inflammatory responses within the airway can have important consequences, particularly in equine athletes.[171,172] Racehorses commonly develop airway inflammation during training, with prevalence rates as high as 70%-80%.[173,174] Lung macrophages may be the primary or secondary site of infection and/or the source of inflammatory cytokines in several important infectious and non-infectious equine diseases, including equine arteritis virus, equine influenza, equine herpesvirus 2 and both mild to moderate equine asthma (MMEA) and SEA.[65,159,175-177]

Moreover, a few studies have assessed the influence of training on immune cell function specifically within the lung, a key consideration in light of the recently reported disassociation between the response of equine monocytes and AMs to training.[178] Both an exercise-associated reduction in equine AM phagocytic capacity[179] and a training-associated derangement in the responsiveness of equine AMs to various TLR ligands have been reported, theoretically reflecting an increased susceptibility to opportunistic infection.[178] In agreement, we recently demonstrated a training-associated alteration in equine AM basal gene expression, which was also consistent with a degree of immunosuppression at the level of the airways.[180]

Bacteria, such as *Streptococcus zooepidemicus*, *Streptococcus pneumoniae* and *Pasteurella/Actinobacillus species*, constitute opportunistic pathogens in the equine airway, triggering the respiratory immune system and inducing inflammation.[181,182] Exposure to other factors, such as *Aspergillus fumigatus* and hay dust, have also been shown to induce an inflammatory response via the activation of AMs.[183] LPS, a significant component of organic dust derived from equine bedding and forage, is considered a major factor in the induction of airway inflammation in stabled horses.[183] Werners and Bryant[184] reviewed the limited literature relating to structure-function relationships among pattern recognition receptors (PRRs) in horses. Early studies found that equine AMs produced large amounts of TNF in response to LPS, but were significantly less responsive to bacterial LPS than blood monocytes, requiring 100-fold higher concentration to induce procoagulant activity.[84] Subsequently, Suri et al[185] demonstrated that TLR4, but not TLR2, is constitutively expressed in healthy horse lungs, with TLR2 being induced by LPS in IMs, a finding also reported in murine, human and porcine macrophages.[12,186,187] It is likely that increased expression of *TLR2* is a consequence of TLR4 signalling in response to LPS, as shown in mice.[188] More recently, Waldschmidt et al[189] reported that, in contrast to equine skeletal muscle tissue cells, equine AMs responded efficiently to TLR2, 3 and 4 ligands. Furthermore, AMs show high expression of the specific macrophage markers CD14, CD163, TLR2, TLR3 and TLR4, have high phagocytic activity and are activated when stimulated with various proinflammatory ligands, thus supporting the importance of the local microenvironment in the activation status of the macrophage.[20] TLR9, the receptor that recognises unmethylated CpG oligodeoxynucleotide DNA,[190] is expressed by equine lung cells, including interstitial and AMs, with its expression also being upregulated by LPS stimulation.[191] Similarly, TLR9 is expressed in mouse and human lung macrophages; its expression is also being enhanced by LPS.[192,193]

Whereas normal mouse and human lungs have no PIMs, they are detected in the capillary endothelium of horses and other species,

such as cattle, pig and sheep, and are recognised as a member of the MPS.[150,160,194,195] Equine PIMs are considered proinflammatory cells that play a critical role in equine lung inflammation, since they secrete the proinflammatory cytokines, TNF and IL-1B, and phagocytose in response to LPS.[196] Depletion of equine PIMs by gadolinium chloride resulted in a decreased severity of LPS-induced lung inflammation, reflected in a reduced mean pulmonary arterial pressure and lower IL-1B production in PIM-depleted horses compared to controls.[196] Furthermore, depletion of PIMs in horses affected with SEA resulted in a reduction in both clinical symptoms and lung inflammation, characterised by a reduced airway neutrophil count and a decrease in *IL8* and *TLR4* mRNA detected in airway-derived cells.[197] Equine PIMs have been shown to express TLR4 and TLR9, and the expression of TLR2, TLR4 and TLR9 in horse lungs was augmented after LPS treatment.[185,191] As PIMs are capable of directly responding to inflammatory stimuli within both the airway and intravascular compartments, they have a unique role in pulmonary immunity, perhaps explaining the recognised susceptibility of the horse to endotoxaemia.[150] Recently, Harrison et al[198] reported an increased expression of inflammatory molecules, such as TLR4 and TLR9, in mononuclear cells in the lungs of septic foals, potentially including PIMs (see Figure 3). This group also reported an increase in PIMs from foals that died from sepsis, compared to healthy animals. Although PIMs are not observed in healthy humans, there is evidence of their existence in patients with liver disorders.[199-202]

4 | THE HORSE AS AN ANIMAL MODEL FOR HUMAN DISEASE RESEARCH

The mouse is the most widely used animal model for studying human disease; however, many differences exist between mice and humans.[13,203-205] As well as the obvious environmental, anatomical, pathophysiological and genetic differences, there are many distinctions in their innate immune responses,[12,60,203,204,206] with specific relevance to mechanistic differences in common inflammatory conditions, such as trauma, burns or endotoxaemia.[13] One such difference between rodents, humans and horses is their sensitivity to LPS, with horses and humans both having a relatively greater sensitivity than rodents. The estimated lethal dose of LPS in humans is thought to be approximately 0.015-0.03 μg/kg[207,208] and 50-200 μg/kg in the horse.[209] In contrast, the LD50 dose in the mouse is 25.6 mg/kg.[210] Despite the clear differences between horses and humans with respect to LPS lethal dose, the fact that this difference is significantly less than that between mice and humans suggests that the horse may be a comparably more appropriate model to study the response to LPS in humans. Horses, like other daylight-active animals including humans and pigs, have evolved differently to nocturnal animals, such as the mouse. Comparisons between the LPS-induced gene expression of equine AMs and BMDMs and that of other species have revealed significant similarities with human-derived cells and significant differences with murine-derived cells, further supporting the potential suitability of the horse as a model of human innate immune responses, both systemically and at the level of the lung.[20-22] Further investigation of these interspecies similarities is clearly warranted; for example, via Cap analysis gene expression or RNA-seq. Findings derived from such studies could provide further insights into the inherently high level of sensitivity to LPS shared by both humans and horses. In turn, this may facilitate the identification of novel therapeutic targets for both endotoxaemia and sepsis in man, an objective which has been met with limited success via the use of rodent models. Similar studies have already been performed in other large animal models such as pig and sheep.[60,61,211]

The Lipid A component of LPS is detected by macrophages via the PRR, TLR4, and the co-receptors, CD14 and MD2. The Lipid A component of LPS varies in structure between different bacterial species. This results in a variation in TLR4-mediated host responses.[212] These variations in Lipid A structure also result in interspecies differences in ligand recognition. For example, the Lipid A from *Salmonella enterica* serovar *Typhimurium* is an agonist in all species studied. The Lipid IVa structure of *Escherichia coli* is a partial agonist in horses,[213] whereas in humans it is a full antagonist,[214] and a full agonist in mice.[215] Similarly, differences in *Rhodobacter sphaeroides* Lipid A recognition also exist between mice, humans and the horse.[216] Likewise, there are species differences in the recognition of lipopeptides by the PRRs TLR1 and TLR2.[217] TLR2 recognises bacterial antigens by forming heterodimers with TLR1 or TLR6 in both humans and horses. Although the response of TLR2/1 to the synthetic bacterial lipopeptides Pam2CSK4 and Pam3CSK4 is different between horse and human, the recognition of Pam2CSK4 by TLR2/6 is analogous between these species.[217] Walsh et al[213] found sequence differences in the MD2-TLR4 complex between species. A consequence of these differences was variation in the local charge distribution on the MD2-TLR4 complex. This suggests that the ability of Lipid IVa recognition, and subsequent signal transduction, is governed by electrostatic forces, with only partial contact between the ligand and MD2-TLR4 complex potentially resulting in reduced signalling; such a phenomenon may explain the interspecies differences in response to Lipid IVa.

Nitric oxide, a product of inducible NO synthase, is a major effector secreted by activated macrophages in rodents; however, it is not produced by human or porcine macrophages.[21,60,203] Instead, LPS-stimulated human and porcine macrophages metabolise tryptophan through the induction of indoleamine dioxygenase (encoded by the *IDO* gene), kynurenine hydroxylase and kynureninase; in contrast, mouse macrophages do not use this pathway.[60,218,219] Among ruminant species, cattle and water buffalo macrophages produce detectable NO and inducible *NOS2* mRNA, whereas sheep and goat macrophages do not.[21] This difference was associated with insertions of an LPS-responsive mobile genetic element in the NOS2 promoter. While Hammond et al[220] reported an increase in *NOS2* mRNA in equine AMs after LPS challenge, with the consequent proposal that the *NOS2* enzyme might be a therapeutic target; others have failed to replicate this finding.[221] Equine alveolar and BMDMs did not produce detectable NO, nor express *NOS2* mRNA or other transcripts required for NO production in response to LPS.[20,21] This

TABLE 1 Comparisons of horse and mouse models for human diseases

Comments		Horse	Mouse	References
General	Body, organ, litter size, longevity	Closer to human	Less similar	93,245
	Breeding costs	High	Low	
	Sample population, possible experiment repetition	Small	Big	
	Availability of immunological tools/ reagents	Limited	High	
Genetic	Genetic diversity	High	Low	244,251-253
	Homology of protein coded genes with human	High	Low	
	Human CD16 is replicated	Replicated	Not replicated	
	Syntenies with human	High	Low	
	Chromosome conservation with human	High	Low	
	Genome annotation	Poor	High	
Macrophage Biology	$CD14^+CD16^+$ monocytes observed in humans	Detected	Not detected	14-21
	NO production in macrophages in response to LPS	Similar	Less similar	
	Response to LPS	Similar	Less Similar	21,22
Diseases	>100 hereditary conditions served as models for humans	Yes	No	1-3,6,7,9,10,74,265-268
	Musculoskeletal disorders-osteoarthritis	Common	Less common	
	Human endotoxaemia-sepsis pathophysiology	Similar	Less similar	
	Asthma—chronic obstructive pulmonary disease pathophysiology	Similar	Less similar	
	Human exercise pathophysiology/ immunology	Similar	Less similar	
	Human infectious diseases	Similar	Less similar	
	Human mental and behavioural disorders	Similar	Less similar	
	Human metabolic diseases	Similar	Less similar	
Therapeutic strategies	Translational application to humans	Possible	Less possible	6,10,269,270

difference between species is associated with promoter evolution. There is clear conservation between the human and horse promoters and the poor alignment of the promoter sequences between mouse/horse and mouse/human.[20,21] Finally, the comparative analysis of LPS-induced global gene expression of equine AMs with that of other species revealed significant similarities with human-derived AMs and significant differences with murine-derived cells, supporting the potential suitability of the horse as a model of human lung inflammation and endotoxaemia.[22]

The full clinical significance of these differences is not yet fully understood; nonetheless, there is clear evidence of interspecies variation in innate immunity. Such differences likely influence the relative magnitude of the response of horses to LPS, or indeed other pathogen-associated and damage-associated molecular patterns. Such interspecies differences have resulted in an ever increasing demand for novel animal models (eg pig) in the study of various human diseases; namely those which more closely resemble human pathophysiology, compared with rodents.[187,222,223] In comparison to rodent models, the use of horses as appropriate models for the generation of data applicable to human disease research has clear practical, financial (eg feeding, housing and handling) and genetic limitations. However, there are also significant advantages of this approach, as proposed by other authors (summarised in Table 1).[2,6,10,224,225]

Horses are animals with a high monetary value, and which require considerable investment from their owners. This is particularly true of equine athletes, whereby morbidity of any kind can result in substantial financial losses. Horses can suffer from an extensive range of infectious and inflammatory diseases, some of which are zoonotic, others of which share certain clinical and pathological features with equivalent human diseases.[226-228] For example, POI is a life-threatening complication in horses,[131] but is also a significant clinical issue

in humans. Furthermore, racehorses resemble elite human athletes and can suffer similar consequences in terms of repetitive injuries and exercise-associated pathology, including arthritis and respiratory tract infections. For example, racehorses commonly develop neutrophilic airway inflammation (MMEA) during the early phase of training.[173,174] MMEA significantly impairs athletic performance[171] and has a clear association with entry into training, with a reduction in prevalence as training progresses.[229,230] Although entry into race training may result in increased airborne exposures to infectious and noninfectious agents, it is also associated with a significant increase in exercise intensity and frequency. The well-recognised association between high intensity exercise and symptoms of respiratory infection among human athletes has fuelled interest in the impact of training on immune function. This phenomenon, known as the '*open window*' theory, reflects a temporal association between intense exercise and increased susceptibility to opportunistic infection.[231,232] Despite localisation of inflammation to the airways in MMEA,[171] few studies have assessed the influence of training on immune cell function specifically at this anatomical site, a key consideration in light of the previously mentioned disassociation between the response of equine monocytes and AMs to training.[178,180]

As with many domesticated animals, selective breeding of horses has produced an array of breeds with specific traits relating to appearance (size and colour), temperament and performance (speed, strength and gait), resulting in more than 450 different breeds with a significant degree of diversity.[233,234] Given the nature of line-breeding, each breed is likely to harbour breed-associated naturally occurring mutations, which may be associated with enhanced susceptibility or resistance to various diseases, many of which share characteristic features with specific diseases in humans. In light of these potential commonalities, the use of technologies, such as next-generation sequencing and genome-wide association studies, could facilitate the detection of quantitative trait loci or even specific genes involved in disease susceptibility. Furthermore, there are potential benefits of using genetic studies in horses in order to study human diseases. Indeed, more than 130 equine hereditary traits relating to specific equine diseases and disorders have already been suggested as valuable models for the study of human equivalents,[235] (OMIA: https://omia.org/home/).

Genetic predisposition to equine respiratory tract diseases has recently been reviewed by Gerber et al.[236] In this respect, SEA (*syn*. recurrent airway obstruction), a common equine respiratory disorder and a recognised model for human asthma, dominates the field of interest with certain chromosomal regions having already been linked to SEA and several candidate interleukin genes having been detected in these regions.[237,238] *IL4RA*, a polymorphic gene involved in the Th2 response, has been recognised as a major candidate gene related to both human asthma and SEA susceptibility.[239] Nonetheless, a few contradictory results have already been published regarding the role of this gene, suggesting loci heterogeneity for such conditions and highlighting the dual effect of genetic background and environmental factors on their development.[237,239,240] These findings reflect the complex pathogenesis and pathogenetic heterogeneity of both the human and equine forms of asthma, that in many cases has been characterised by a mixed as well as a polarised T helper cell immune response.[241-243]

Many genes involved in immune function have a greater level of similarity between human and horse, than between human and mouse.[244-246] Milenkovic et al[244] identified 113 conserved segments between the equine and human genome. For example, *IL2* showed 72% identity with human and both *IL23* and *IL17* showed greater nucleotide sequence identity with human (89% and 84% respectively) than mouse (77% and 75% respectively).[246] Similarly, equine IFN-γ-induced chemokine *CXCL9* has 86% homology with human, but only 74% with the mouse.[247] Moreover, it is evidenced that CD14 as well as TLRs 2-5 and 9 are highly conserved between human and horse (Figure 5). With specific relevance to macrophages, equine *CSF1R* is similar to human *CSF1R*, containing 21 exons which code a 968 amino acid protein within a 30 kb region.[248-250] Steinbach et al[245] reported on the capacity for equine CSF2 to induce proliferation of a human TF-1 cell line and also demonstrated cross-reactivity of antihuman CD14, CD163 and CD206 monoclonal antibodies against horse PBMC. Furthermore, the myeloid differentiation marker ADGRE1 (F4/80) has recently been shown to be expressed by equine BMMs.[62]

Chowdhary et al[251] were the first to study the radiation hybrid map of the horse genome and found the level of human-horse synteny to be greater than the level of mouse-horse synteny. While approximately half the number of horse chromosomes showed conserved synteny to one human chromosome;[235] gene homology is still highly conserved between species.[252-254] For example, Raudsepp et al found that the majority of equine chromosomes show homology to human chromosomes; the same group later demonstrated high conservation of gene order between horse and human chromosome X. These findings increase the likelihood that discoveries derived from the horse will have direct benefits for both equine and human health.

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

In agreement with data derived from other species, the observations on the equine MPS summarised in this review refute the concept that 'a macrophage is a macrophage', highlighting the importance of studying cells derived from the specific tissue of interest. Recent global analyses on macrophages derived from mouse, human, pig, sheep and water buffalo have confirmed that tissue macrophages from different anatomical locations differ radically in their gene expression profiles;[61,211,255-257] currently, there are a limited number of transcriptomic studies highlighting tissue differences in the horse. Graham and co-workers initiated the investigation of the horse transcriptome using human microarrays on equine brain and liver tissues and articular chondrocytes.[258] Other studies using microarrays[22,259] and RNA-seq[260] investigated the equine transcriptome of various tissues. Although generally not designed to specifically assess the level of tissue macrophage differentiation, extraction and analysis of

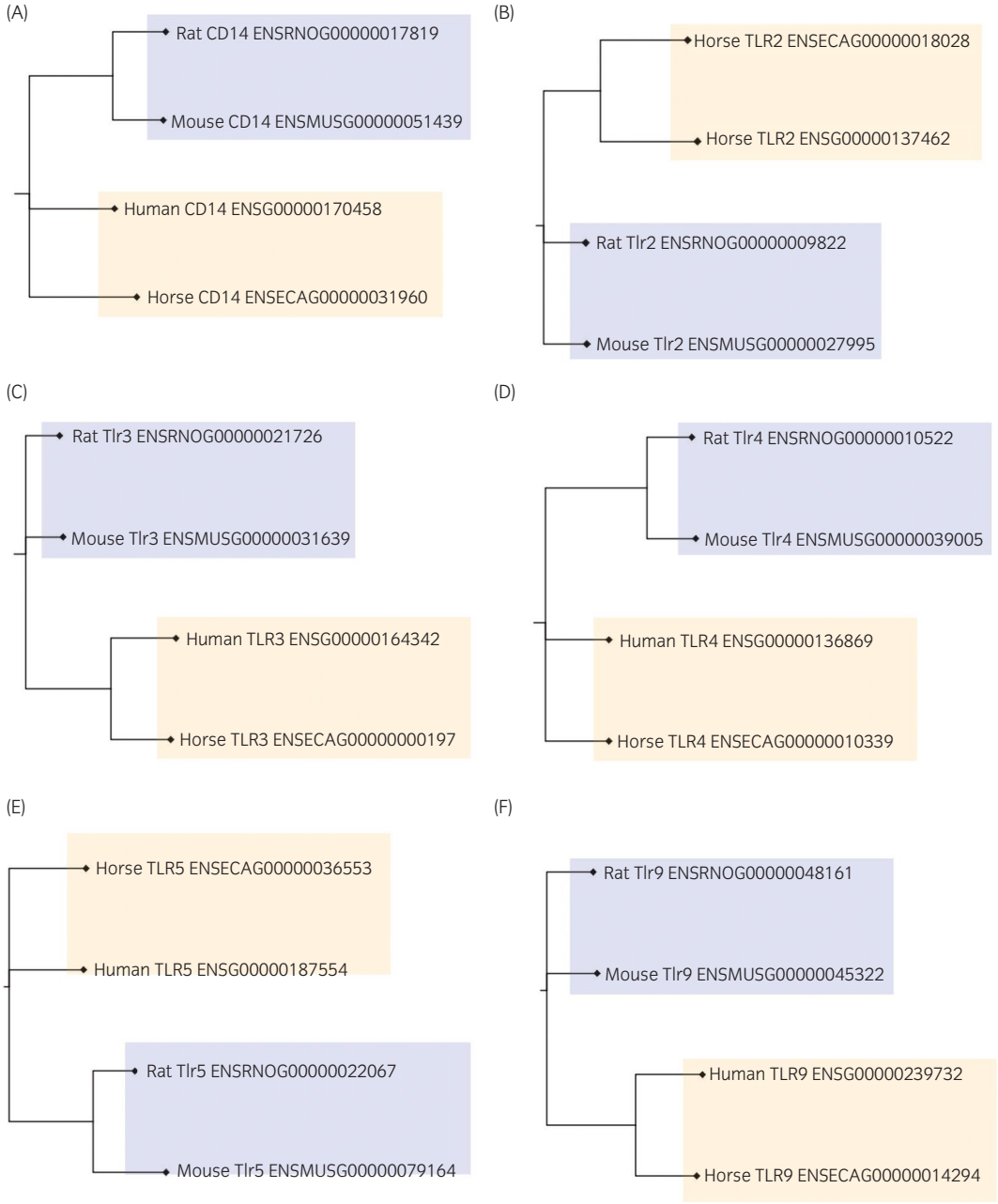

FIGURE 5 Conservation and divergence of immune genes of the horse—comparison with humans and rodents. Phylogram trees of the innate immune response genes *CD14* (A), *TLR2* (B), *TLR3* (C), *TLR4* (D), *TLR5* (E) and *TLR9* (F) of rat, mouse, human and horse. Sequences were obtained from www.ensembl.org (Release 100).[271] Analysis was generated by Clustal Omega[272] and FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Each gene sequence is presented by species' official gene name and Ensemble gene ID

data derived from these studies clearly demonstrated tissue-specific expression profiles which correlate with specialised tissue functions. Further expansion of this work is warranted, including the genomic, phenotypic and functional characterisation of enzymatically dissociated macrophage populations derived from different tissues (eg brain [microglia], liver [Kupffer cells], spleen (*already isolated in our lab*) and GIT). Furthermore, the relatively recent development of equine tissue banks, such as the Equine Respiratory Tissue bank, University of Montreal (http://www.btre.ca/media/html/en_biobank.html) and the extensive Thoroughbred-derived biobank described by Burns and Roberts (2018), should greatly facilitate the generation of data with cross-species translational potential.

Despite extensive research on equine inflammatory diseases, specific information on the equine MPS is lacking, largely due to the limited availability of appropriate molecular reagents. Consequently, many previous studies have relied on the successful use of cross-reactive human reagents.[93,245] With the development of genomic/transcriptomic resources for the horse[234] and emerging data from

numerous other large mammals (human, pig, cattle, sheep and water buffalo), it is now increasingly possible to address what is known, and what is not known, about the innate immune system of the horse in comparison to other species. The equine reference genome, EquCab2, from the Thoroughbred mare *Twilight* was first released in 2007 and advances in genomic technologies led to a new assembly of the reference genome, EquCab3, in 2018 using long-range sequencing data to improve contiguity.[261] While the equine reference genome has led to developments in equine genomics,[235] equine genomic annotation still remains challenging[234,262,263] and a high-resolution atlas of equine gene expression would substantially improve future efforts in this field.

Justification for the proposal of the horse as an appropriate animal model for human macrophage biology would necessitate the global analysis of gene expression across multiple tissues in this species. The need for defining the tissue-specific gene expression, regulation and functional annotation across domestic animal species has already been acknowledged by the establishment of the International FAANG project.[264] The completion of the equine genome annotation would provide a major tool for future transcriptomic horse studies, the findings of which could selectively be applied to humans.

Together, in addition to the important similarities in both pathophysiology and macrophage/monocyte biology between horses and humans, the horse consists a great source of large volumes of different kind of samples that could be used for future studies. The volume of equine samples and the associated cell retrieval rates represent at least 2-3 orders of magnitude greater than those obtained from rodent models. Thus, for all these reasons highlighted above, we believe that greater consideration should be given to the horse as an appropriate candidate model for human disease.

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CONFLICT OF INTEREST

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